VOL. XII, No. 6 JUNE, 1951

JUL 16 1951

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BORDEN'S REVIEW

of

NUTRITION RESEARCH

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BORDEN'S REVIEW
of
NUTRITION RESEARCH

Reviews and summaries of research information concerning nutrition and foods. Published monthly, except July and August, by THE BORDEN COMPANY; officers of the Company: Theodore G. Montague, president; E. L. Noetzel, treasurer; T. D. Waibel, secretary. Editorial office: 350 Madison Avenue, New York 17, N. Y.

THE CITROVORUM FACTOR

By RUTH WOODS

In the six years following the discovery of folic acid (1945) and of its clinical effectiveness in stimulating hematological responses in pernicious anemia and other types of anemias (1), a host of new developments have followed. These have included the special roles of thymine and thymidine (2) and of vitamin B_{12} (3) as anti-anemia factors; the limitations of folic acid with respect to correcting the neurological complications of pernicious anemia; the discovery of folic acid antagonists and their possible value in the treatment of the leukemias; and finally, the discovery of a whole new group of compounds — both natural and synthetic — closely related to folic acid.

These new compounds, described variously as the "citrovorum factor" or "folinic acid complex," have been found to possess folic acid activity for both microorganisms and man, and in some cases, to surpass folic acid in growth-stimulating and other properties. The new folic acid derivative has also been found to be many times more effective than folic acid itself in reversing the toxicity of folic acid inhibitors for microorganisms, and there are some indications that it may act similarly in man. This last has opened up a new therapeutic possibility in the treatment of human leukemias in which conditions folic acid antagonists have been found to be of some value in inducing temporary remissions. Up until now, clinical work with folic acid antagonists has been somewhat unsatisfactory because of the toxic side reactions which accompany their use and which are not counteracted by folic acid, even in large doses. Folinic acid, however, has been found to be very effective in both preventing and reversing these toxic reactions in man, so that it promises to be an invaluable factor not only in the direct control of the anemias, but in the indirect therapy of the leukemias as well.

Early Studies Leading to Discovery of the Citrovorum Factor:

During a survey of microorganisms which could be used in the microbiological determination of amino acids, particularly alanine, Sauberlich and Baumann (4) found that the organism *Leuconostoc citrovorum* failed to grow on the customary synthetic medium suitable for most other assay organisms. *Leuconostoc citrovorum* was found to grow only upon the addition to the medium of certain crude liver extracts or of certain commercial liver preparations used in the treatment of pernicious anemia. In the pres-

ence of these supplements, the organism grew satisfactorily and could be used successfully for the microbiological assay of alanine and other amino acids.

These observations led to a new line of inquiry, namely the investigation of the nature of the unidentified factor in liver concentrates which was essential for the growth of L. *citrovorum.*

Folic Acid: Addition of a commercial liver preparation* containing the anti-pernicious anemia principle (i.e., vitamin B_{12}, at that time just isolated but not yet available) induced extremely rapid growth of the microorganism. Addition to the medium of thiamine, pyridoxal, pyridoxamine, etc. had no effect on the growth response to the liver fraction. Addition of large amounts of folic acid, however, caused a further growth response, as measured in terms of acid production.

The response of the organism to folic acid, however, differed qualitatively from that elicited by the unknown factor in liver: there was a marked delay in the growth of the organism when it was given folic acid alone, and no visible growth was noted during the first 24 to 36 hours. Further, the amount of folic acid contained in the liver concentrate was far less than that already contained in the basal medium, so that the response of the organism to the liver concentrate could not be attributed to its folic acid content.

Other Factors: Some growth was obtained with certain other liver concentrates, peptone or a crude yeast extract, as well as with pure thymidine. The response to thymidine, like that to folic acid, however, differed considerably from that induced by the active liver preparations. All liver concentrates known to be active against pernicious anemia were also active for L. *citrovorum,* but a 30-fold variation was found between preparations in the ratios of microbiological activity to clinical potency. This indicated, apparently, that the activity of the liver fractions was due to a component other than the anti-pernicious anemia principle (vitamin B_{12}). The partial ability of thymidine and the complete effectiveness of the liver factor to promote growth of L. *citrovorum* was similarly reported by other groups of investigators about this time (4a, 4b).

Sauberlich and Baumann recognized the relation of the unidentified factor to folic acid and pointed out the interesting similarity between the behavior of both L. *citrovorum* and of pernicious anemia patients with respect to their response to high levels of folic

*Reticulogen, Lilly

acid cause a remission of a part of the symptoms associated with pernicious anemia, but do not effect the complete cure that is obtained with crude liver concentrates. Similarly, high levels of folic acid stimulated growth of the microorganism, L. *citrovorum,* but in a delayed manner quite different from that obtained with active liver concentrates.

In further studies (5) the response to folic acid, as well as to thymidine, was confirmed. In addition, with the availability of crystalline vitamin B_{12}, it was demonstrated, in confirmation of earlier suppositions, that the *citrovorum* factor is distinct from the anti-pernicious anemia factor. Fractionation of the active principle yielded concentrates which were more active by weight than either folic acid or thymidine (5). The active *citrovorum* factor in these concentrates obviously bore some relation to folic acid and thymidine, and possibly to vitamin B_{12} as well, but what the nature of this relationship was necessitated further research.

Interrelationships of Citrovorum Factor, Folic Acid, Thymidine and Vitamin B_{12}:

Folic Acid: In an attempt to assess the relationship of folic acid to the new *citrovorum* factor (CF), Sauberlich (6) studied the effect in rats of folic acid supplements on the urinary excretion of the *citrovorum* factor. Rats fed the basal diet without supplementary folic acid excreted an average of 140 CF units per day. Animals fed a daily supplement of 10 micrograms of folic acid, on the other hand, excreted an average daily amount of 20,000 CF units. With folic acid supplements of as little as one microgram per day, urinary excretion of the CF was still markedly increased, averaging about 2000 *citrovorum* units.

(Role of Intestinal Microorganisms): Sauberlich (6) demonstrated further that interference with vitamin synthesis of intestinal microorganisms through supplementary feeding of sulfaguanidine does not appreciably diminish the *citrovorum* activity of the urine. This would seem to indicate that intestinal synthesis is not a factor in the production of urinary *citrovorum* activity. This fact was further substantiated by the increased urinary activity resulting from folic acid administration by injection, as well as by oral feeding.

Estimation of the folic acid content of the urine, following feeding or injection of folic acid supplements, showed that the amounts present were not sufficiently large to account for the increased stimulation of growth of the test organism, L. *citrovorum,* thereby eliminating folic acid itself as the factor responsible for the urinary activity.

(Human Study): A single study was conducted with a human subject with results similar to those obtained in the rat studies. Before the ingestion of extra folic acid, the subject excreted about 6500 *citrovorum* units per day; following a single dose of 30 mg. of folic acid, the excretion rose to 425,000 units in the following 24-hour period.

As a result of these observations, Sauberlich concluded that (1) either folic acid is converted in the body to a more active compound, or that (2) the added folic acid stimulates the excretion of an active compound. The resulting product may actually be the metabolically active form of folic acid animals or it may be merely an excretory product of folic acid metabolism.

Related Factors: Close metabolic relationships have been indicated among folic acid, vitamin B_{12}, thymidine and ascorbic acid (7–16). These have been described in considerable detail in previous Reviews (2, 17) and will not be repeated here. Suffice it to mention that thymidine, for example, has been shown to be capable of replacing the vitamin B_{12} requirement for certain bacteria (9, 11, 12, 13, 14) and, in some cases, the folic acid requirement as well (12). Sauberlich's studies similarly showed a limited ability of thymidine to replace the factor required by L. *citrovorum* (4). Supplements of thymidine, vitamin B_{12} or ascorbic acid to rat diets, however, had no effect in increasing the CF activity of the urine in a manner comparable to that effected by the feeding of folic acid (6).

Relation Between Folic Acid Antagonists and Citrovorum Factor:

Indications of a significant interrelation between the *citrovorum* factor and folic acid were greatly strengthened as a result of inhibition studies involving folic acid antagonists. Thus, for example, in the studies of Shive *et al* (8) it was noticed that while methylfolic acid (antagonist) interfered with the growth of certain microorganisms, this toxicity was overcome more effectively by thymidine than by folic acid itself. In later studies by Sauberlich (18) it was found that either methylfolic acid or aminopterin* prevented growth of L. *citrovorum* when cultured on a complete medium (including folic acid) in the presence of controlled amounts of liver concentrate (Reticulogen, Lilly—source of CF). Extra folic acid failed to counteract this toxicity, as did also vitamin B_{12}. Extra amounts of reticulogen or of the *citrovorum*—active concentrate from rat urine, however, were extremely effective—even in small doses—in permitting resumption of growth in the presence of the folic acid antagonists.

*4-Aminopteroylglutamic acid (4-amino PGA)

Extremely large amounts of folic acid were able to counteract the inhibitor, about 300 micrograms of folic acid being required to overcome the effect of only 2 micrograms of aminopterin. Reticulogen was from 100 to 600 times more effective than folic acid (1 microgram reversed the effects of from 1 - 4 micrograms of aminopterin). Thymidine, also, was somewhat effective, being considerably more so than folic acid, but not as potent as the liver concentrate. Chromatographic analyses of both liver concentrate and urine concentrate showed almost complete absence of thymidine from these, thereby eliminating thymidine as the possible factor responsible for the activity of these materials.

At this point at least one fact was clear: the *citrovorum* factor was obviously related to folic acid in some way. The fact that large supplements of folic acid led to increased concentrations of the CF in the urine; that folic acid, in large amounts, could partially replace the factor in liver (*not* vitamin B_{12}) for growth purposes in L. *citrovorum;* and finally, the fact that the *citrovorum* factor was far more effective than folic acid itself in counteracting the toxic effects of folic acid antagonists, seemed to lead to one conclusion as to the nature of this relationship. This was that the *citrovorum* factor must be a more active form of folic acid itself, rather than a new vitamin or growth factor — probably derived from folic acid through specific enzymatic conversion. It was also apparent that some organisms can accomplish this conversion of folic acid to *citrovorum* factor more readily, whereas others, such as L. *citrovorum,* cannot do so easily and thus require either the more active form, already pre-formed, or a tremendous supply of the precursor (folic acid).

Conversion of Folic Acid to Citrovorum Factor:

The theory proposing the conversion of folic acid to *citrovorum* factor was put to the test by Nichol and Welch (19) in a series of experiments investigating the ability of rat liver slices to convert folic acid to CF. They obtained liver slices from animals in whom severe folic acid deficiency had been induced by the combined effects of a folic acid-free diet, plus the feeding of a sulfa drug, for a period of five weeks. These slices, following preliminary preparation, were incubated alone, with addition of folic acid, with addition of ascorbic acid, and with addition of both folic and ascorbic acids.

They found that liver slices from normal rats contained appreciable amounts of CF, whereas livers from folic-acid deficient animals were extremely low in CF (from 36 to 147 units of CF per gram in deficient livers as compared with about 1700 to 2880 in normal livers). When these livers

were incubated with folic acid, the CF concentration in both normal and deficient livers reached approximately the same high levels — equal to about a 100 per cent increase for the normal livers and a 2000 to 10,000 per cent increase for the deficient livers.

Role of Ascorbic Acid: Because of the many indications, both experimental and clinical, of the role of ascorbic acid in certain megaloblastic anemias — and of its possible metabolic interrelationship with folic acid — further studies were made on the effect of ascorbic acid supplements in the conversion of folic acid to *citrovorum* factor by liver slices. It was found that when normal liver slices were incubated in the presence of ascorbic acid, the CF activity was doubled just as it had been when extra folic acid was provided. Incubation of normal liver slices with both folic and ascorbic acids resulted in a three- to five-fold increase in CF activity.

When liver slices from folic acid-deficient rats were incubated alone, only a slight amount of *citrovorum* factor was present or produced. The addition of ascorbic acid exerted little effect on the yield of *citrovorum* factor. But when folic acid, alone, was added, the CF activity rose to levels approximately those of normal livers incubated with folic acid. Incubation of deficient liver slices with both folic and ascorbic acids doubled this amount of CF.

These findings clearly indicated that folic acid is essential to the formation of the *citrovorum* factor and that this conversion is remarkably augmented by the presence of ascorbic acid. In this connection, Nichol and Welch point out:

> "The fact that ascorbic acid increases the formation of CF from PGA* suggests the possibility that a severe deficiency of vitamin C might depress the metabolic alteration of PGA to CF. In keeping with this possibility is the demonstrated relationship of scurvy to the appearance of megaloblastic anemia in human infants and monkeys—a condition relieved by treatment with PGA.
>
> "Also, in another species unable to synthesize ascorbic acid, the guinea pig, a deficiency of vitamin C leads to an abnormality in the metabolism of tyrosine** —a condition also relieved by relatively large doses of PGA.
>
> "It is reasonable to postulate that the conversion of PGA to CF is limited by a severe deficiency of ascorbic acid, so that effective amounts of the essential derivative are no longer obtained from PGA unless the intake of folic acid is materially increased."

Folinic Acid:

While the studies with the *citrovorum* factor were in progress, another group of investigators independently reported the discovery of a new group

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*PGA, i.e. pteroylglutamic acid, i.e., folic acid

***cf. Borden's Review of Nutrition Research,* September, 1949, "Dietary Interrelationships—Vitamin C and the Metabolism of Aromatic Amino Acids."

of vitamin factors related to folic acid. These workers, Bond, Bardos, Sibley and Shive (20) were engaged in inhibition analysis studies of factors functionally related to para-aminobenzoic acid and folic acids. They found that while members of the folic acid group were capable of reversing the toxicity of the antimetabolite, methylfolic acid (for the organism, L. *casei*), crude or refined liver extracts proved about 15 times as active as could be accounted for on the basis of their folic acid content. Further work with these liver fractions yielded a potent concentrate of the new substance which, on the basis of structure and functional relationship to folic acid, was named *folinic acid*. Analyses further revealed the presence of at least two additional related factors in the liver extracts, so that the existence of a "folinic acid group" was postulated. Folinic acid was found to possess growth promoting activity similar to folic acid and to be more potent than folic acid in counteracting the toxicity of folic acid inhibitors.

In other studies, (21) folinic acid was demonstrated to behave similarly to the *citrovorum* factor with respect to its growth promoting properties for L. *citrovorum*. It was further observed that mild acid hydrolysis destroyed the CF activity of the concentrate, but did not impair its folic acid activity. It was, therefore, concluded that CF and folinic acid are probably identical and that the active substance is probably an acid-labile derivative of folic acid.

The synergistic action and interchangeability of folinic acid and thymidine was also noted by these workers. They interpreted this to indicate the involvement of folinic acid (CF) in the biosynthesis of thymidine, as well as the further involvement of thymidine in the biosynthesis of the active form of folic acid (20). Similar conclusions were reached by Broquist *et al* (21a) in independent investigations of the biological and chemical properties of the *citrovorum* factor.

Soon after these studies with the naturally occurring folinic acid, Shive and coworkers (22) reported the chemical synthesis of a similar, if not identical, factor from folic acid through the intermediate synthesis of formylfolic acid. Reduction of formylfolic acid in the presence of ascorbic acid led to the production of a synthetic concentrate identical in physiological properties to the naturally occurring folinic acid concentrate derived from liver.

This was very shortly followed by the synthesis and isolation of this factor in crystalline form by Brockman and associates (23). This material, active for both L. *citrovorum* and for chicks as a growth factor, also was effective in reversing the toxicity of the folic acid antagonist, aminopterin, in mice.

Relation to Nucleic Acid Synthesis:

The interrelationships among vitamin B_{12}, folic acid, thymidine, the *citrovorum* factor and other desoxyribosides are both extremely important and quite complicated. At present, indications are that the factor which they may all have in common is their functioning, in various manners, in the synthesis of the nucleic acids. Before discussing the possible mechanisms whereby these functions may be performed, it may be desirable for purposes of clarity to review briefly the nature and importance of the nucleic acids and their structural components (23a).

Biological Significance of the Nucleic Acids: For protein synthesis in general and for red blood cell synthesis, specifically, nucleoproteins are key essentials. Because these nucleoproteins (which are composed of nucleic acids and proteins) are actively involved in protein synthesis they are also a major factor in the phenomenon of growth.* In all cases in which rapid protein synthesis takes place, such as in embryonic development; in protein-synthesizing tissues (like the enzyme-producing cells of the pancreas, for example); cancer tissue; blood cells, and the like, a direct correlation has been found between such protein synthesis on the one hand and the high content and high rate of turnover of nucleic acid in such tissues, on the other (24, 25, 26, 27). It is of further interest to note that the self-duplicating protein structures which are the keys to biological reproduction and to protein synthesis, all contain nucleoproteins. These structures include the genes, plastids, transforming principles of bacteria, and perhaps also the formed particles of the cytoplasm.

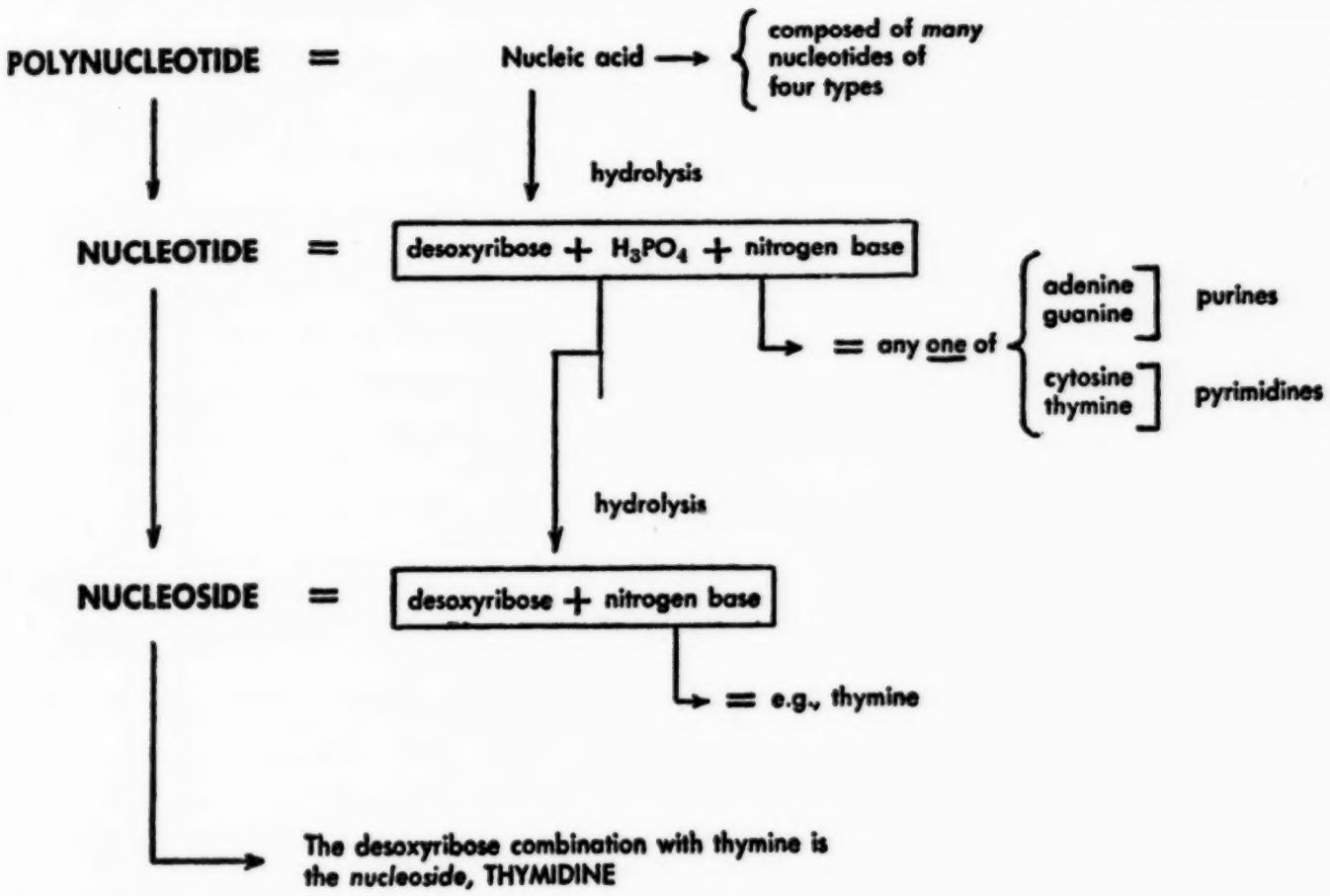
Components of Nucleic Acids: Of all the nucleic acids which have been isolated and studied, from both plant and animal sources, two basic types have been generally recognized: Those characterized by a pentose sugar component (called pentosenucleic acids, PNA) and those which contain a desoxypentose sugar (desoxypentosenucleic acids, DNA). An example of the DNA class is thymus nucleic acid in which the sugar is desoxyribose. Thymus nucleic acid occurs in both plant and animal cells; its chief locus is in the chromosomes.

When DNA is hydrolyzed by dilute alkali, four separate components may be isolated. These are called nucleotides. Each of these nucleotides contains one molecule each of the sugar, desoxyribose; of phosphoric acid; and of a nitrogenous base. The nitrogenous base may be either one of the purines, *adenine* or *guanine,* or one of the pyrimidines, *cytosine* or *thymine.* Thus, the only distinction among the four nucleotides is in the nature of

*Possibly an explanation for the reputed growth-promoting properties of vitamin B_{12} in children.

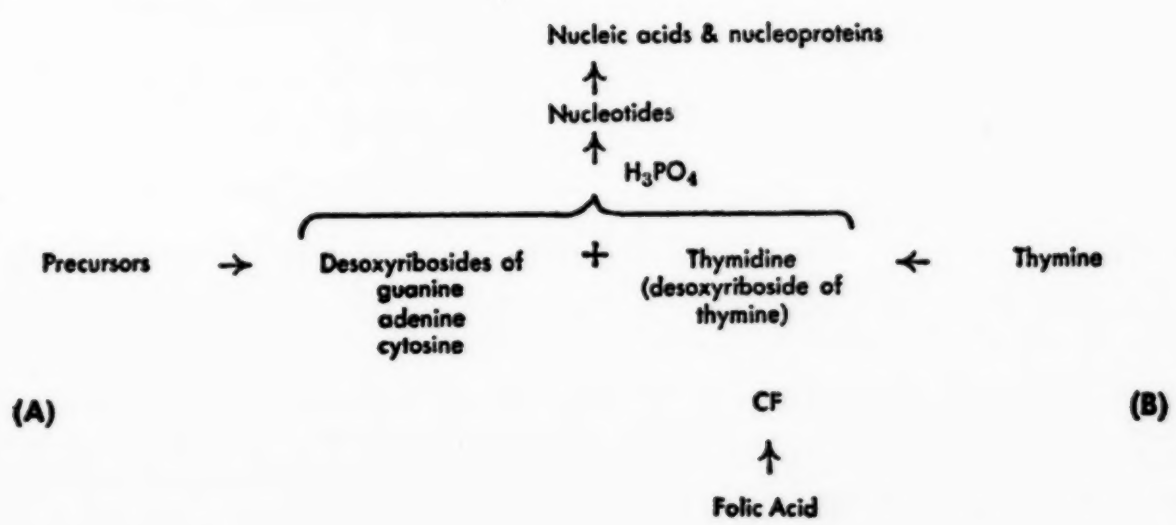
the respective nitrogen base moieties. The nucleotide component is the fundamental unit of all nucleic acids, the nucleic acids themselves being polymers of high molecular weight (i.e. *poly*nucleotides). The phosphoric acid may be hydrolyzed from a given nucleotide to yield a nitrogen base-sugar combination known as a nucleoside or, in this case, a desoxyriboside. Further hydrolysis of the nucleoside yields desoxyribose and the respective purine or pyrimidine (Fig. 1).

Fig. 1 Structure of Nucleic Acid in Terms of Components



Interrelations Between CF and Related Factors and Nucleic Acid Synthesis:

As a result of studies with several micro-organisms, Jukes, Broquist and Stokstad (30) formulated a hypothesis relating vitamin B_{12}, folic acid and the *citrovorum* factor with thymidine and other desoxyribosides. The following is an expanded version of the schematic diagram:



On this basis, Jukes *et al* suggest that L. *leichmanii** would be able, for example, to carry out step (B) but not step (A); conversely, L. *citrovorum** may carry out step (A) but not (B). In support of this they found that on purified media, L. *citrovorum* produced vitamin B_{12} activity, while L. *leichmanii* produced *citrovorum* factor activity. These suggestions would seem to be in line with similar earlier observations of Wright, Skeggs and Huff (11) who postulated that:

> "The primary biochemical defect in pernicious anemia may well be the inability to synthesize certain nucleosides, particularly thymidine, from parent purines and pyrimidines."

It would thus appear that at least significant portions of the biochemical roles of vitamin B_{12}, folic acid and *citrovorum* factor are related to the synthesis of nucleosides (such as thymidine) or nucleotides, and accordingly, of the nucleic acids themselves.

Insofar as this function is concerned, CF seems to be the active principle by means of which folic acid plays a role in hematopoiesis. Folic acid seems to have other functions as well, however, in which CF is not directly a participant. In this latter connection lies the extremely interesting use of CF in the therapy of leukemic diseases. In such therapy, folic acid antagonists, such as aminopterin or a-methopterin, are used to produce apparently effective responses. Toxic side effects resulting from folic acid deficiency due to the presence of such antagonists are not overcome by folic acid itself, however, but through the use of CF.

Because of the greater growth-promoting activity of CF or folinic acid for certain microorganisms as compared to folic acid, it has also been suggested (20) that this new form of folic acid may be of especial value in the clinical treatment of sprue, pernicious anemia and other nutritional deficiencies related to the folic acid and vitamin B_{12} group, in addition to its possible clinical role in the treatment of leukemia.

Conclusion:

The newly discovered *citrovorum* factor, probably identical with both the naturally occurring and synthetic "folinic acid," is at present regarded as a derivative of folic acid. For some organisms this derivative is apparently more active than is folic acid itself. Also, some organisms are able to synthesize the *citrovorum* factor from folic acid — a process which is greatly facilitated by the presence of ascorbic acid — whereas others cannot

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*The desoxyribosides of these purines, as well as of thymine (i.e. thymidine) have been shown to promote growth—substituting for vitamin B_{12}—of L. *leichmanii* (10, 28, 14, 29). On the other hand, *citrovorum* factor and thymidine, but *not* the other desoxyribosides or vitamin B_{12}, permit growth of L. *citrovorum* (28, 4, 4a, 4b).

do so and require CF preformed in the nutrient media. Existing evidence seems to indicate further that at least in hematopoiesis the *citrovorum* factor may be the enzymatically active form of folic acid, much in the same manner as pyridoxal is related to the coenzyme form of pyridoxamine (vitamin B_6) (31).

Further studies on the *citrovorum* factor promise to be of exceptional interest from both the theoretical and practical points of view. Theoretically, elucidation of the mechanism of folinic acid activity will shed further light on the metabolic roles of folic acid, vitamin B_{12} and other factors involved in the anemias; practically, the use of the *citrovorum* factor in clinical work may lead to greater progress in the existing therapy of both anemias and leukemias.

Beginning studies, both experimental and clinical, on the effectiveness of *citrovorum* factor in anemia and leukemia have already appeared and will be described in a near future issue of this Review.

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